

DNA-Catalyzed Lysine Side Chain Modification**

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Abstract: Catalyzing the covalent modification of aliphatic amino groups, such as the lysine (Lys) side chain, by nucleic acids has been challenging to achieve. Such catalysis will be valuable, for example, for the practical preparation of Lys-modified proteins. We previously reported the DNA-catalyzed modification of the tyrosine and serine hydroxy side chains, but Lys modification has been elusive. Herein, we show that increasing the reactivity of the electrophilic reaction partner by using 5'-phosphorimidazolid (5'-Imp) rather than 5'-triphosphate (5'-ppp) enables the DNA-catalyzed modification of Lys in a DNA-anchored peptide substrate. The DNA-catalyzed reaction of Lys with 5'-Imp is observed in an architecture in which the nucleophile and electrophile are not preorganized. In contrast, previous efforts showed that catalysis was not observed when Lys and 5'-ppp were used in a preorganized arrangement. Therefore, substrate reactivity is more important than preorganization in this context. These findings will assist ongoing efforts to identify DNA catalysts for reactions of protein substrates at lysine side chains.

Deoxyribozymes are specific DNA sequences that have catalytic activity.^[1] We have focused on expanding deoxyribozyme catalysis to include reactions of peptide side chains,^[2] with the longer-term goal of achieving DNA-catalyzed covalent modification of large proteins. Our initial report demonstrated robust DNA catalysis (more than 70% yield in 1 hour) of nucleopeptide formation between the nucleophilic tyrosine (Tyr) phenolic OH side chain and an electrophilic 5'-triphosphate RNA (5'-pppRNA; Figure 1a).^[2a] However, catalysis by separate new deoxyribozymes involving the serine (Ser) aliphatic hydroxy side chain was extremely poor (approximately 0.2% yield), and reactivity of the lysine (Lys) amino side chain was not observed. That initial study presented each single amino acid residue in a highly preorganized three-helix-junction (3HJ) architecture, in which the

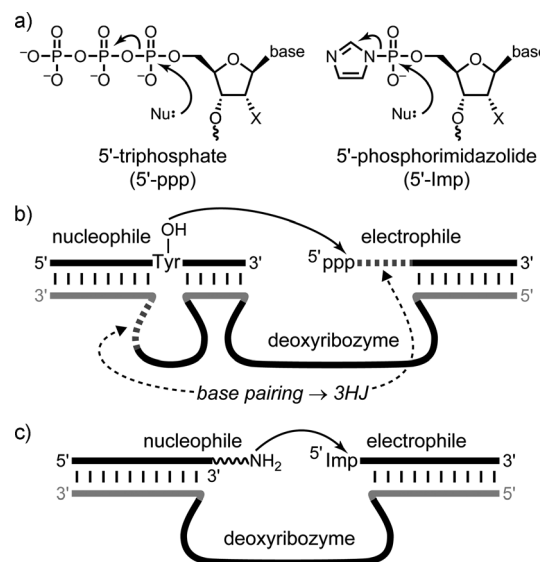


Figure 1. Electrophilic substrates and architectures for DNA-catalyzed nucleophilic reactivity of amino acid side chains. a) Electrophilic substrate structures, showing attack by a nucleophile (Nu:), such as an hydroxy or amino group. X=OH (RNA) or H (DNA). b) Three-helix-junction (3HJ) architecture with Tyr as nucleophile and 5'-triphosphate (5'-ppp) as electrophile. Base pairing creates the third helix and preorganizes the nucleophile and electrophile; see previous studies for origin and analysis of the 3HJ.^[3] c) Open architecture, with amine as nucleophile and 5'-phosphorimidazolid (5'-Imp) as electrophile.

nucleophilic side chain and the electrophilic 5'-ppp are closely juxtaposed (Figure 1b).^[3] We subsequently found that retaining the 3HJ architecture but expanding the substrate to include a tripeptide rather than a single amino acid enabled much more robust Ser reactivity.^[2b] In contrast, when Lys was similarly presented, reaction was instead observed at the oxygen atom of a nearby phosphoramidate linkage, whereas Lys reactivity was still absent.^[2d] This finding suggested that more reactive electrophiles than 5'-ppp should be examined for DNA-catalyzed reactions of amine nucleophiles.

Nature provides significant precedent for protein-catalyzed reaction of the Lys side chain with various electrophiles, including activated acyl groups.^[4] Lys also reacts with the 5'-triphosphate moiety of ATP (adenosine triphosphate) during reactions catalyzed by RNA and DNA ligase enzymes^[5] and in protein adenylation reactions.^[6] In nonbiological contexts, more reactive phosphorus electrophiles than 5'-ppp can be employed. Prebiotically focused nucleotide oligomerization experiments have typically used more reactive nucleotide analogues, such as 5'-phosphorimidazolid (5'-Imp) rather than 5'-ppp,^[7] for which the reactivity difference is on the order of 100-fold. Herein, we evaluated 5'-Imp as a more

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reactive electrophile (Figure 1 a). The less structurally constrained architecture of Figure 1 c was employed in all experiments, because our long-term goal is to identify deoxyribozymes that function with free peptide and protein substrates,^[8] and the 3HJ architecture of Figure 1 b is incompatible with this goal. The DNA-catalyzed nucleophilic Lys reaction was achieved by using 5'-ImpDNA in the less preorganized architecture. From these results, we conclude that DNA can catalyze covalent modification of the nucleophilic Lys side chain, and a high degree of preorganization is dispensable when the electrophile is sufficiently reactive.

In vitro selection^[9] was used to identify deoxyribozymes that catalyze covalent modification of amino groups. In this selection process, random-sequence populations are iteratively enriched through multiple rounds to identify those particular sequences that have catalytic activity. Two amine substrates were used in these selection experiments (Figure 2 a). The first substrate, DNA-C₃-NH₂, presents an aliphatic amino group on a short C₃ tether at the 3'-terminus of a DNA oligonucleotide anchor. The second substrate,

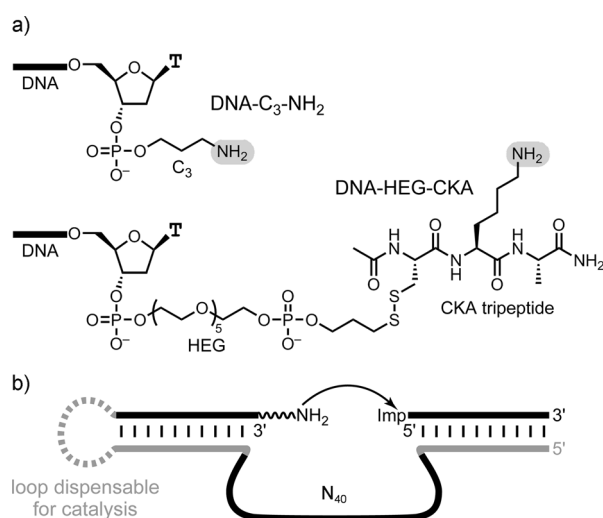


Figure 2. a) Structures of the aliphatic amine and Lys nucleophiles. b) Key in vitro selection step. N₄₀ denotes a 40 nucleotide random-sequence region. The dashed loop enables separation of catalytically active DNA sequences from the random N₄₀ population by PAGE shift but is dispensable for catalysis and was absent in all single-turnover kinetic assays. The PAGE-shifted products were separated and amplified by PCR to enter the next selection round. See Figure S1 for selection details.

DNA-HEG-CKA, presents Lys as part of a Cys-Lys-Ala (CKA) tripeptide substrate covalently attached using a disulfide linkage to a hexaethylene glycol (HEG) tether at the DNA 3'-terminus. For both amine substrates and particularly for the HEG-tethered Lys, the degree of preorganization is low, especially in comparison to the highly preorganized 3HJ architecture (compare Figure 1 c to Figure 1 b).

Many in vitro selection experiments have shown Mg²⁺, Mn²⁺, and Zn²⁺ ions to be effective catalytic cofactors for DNA.^[1b-g] Using the two amine substrates of Figure 2 a, parallel selection experiments were performed using an

N₄₀ random-sequence region (40 random nucleotides). For each experiment, incubation conditions in the key selection step of Figure 2 b were either: (A) HEPES (70 mM), pH 7.5, with Mg²⁺ (40 mM), Mn²⁺ (20 mM), Zn²⁺ (1 mM), and Na⁺ (150 mM) at 37 °C for 14 hours; or (B) CHES (50 mM), pH 9.0, with Mg²⁺ (40 mM) and Na⁺ (150 mM) at 37 °C for 14 hours (all metal ions provided as chloride salts). Neither Mn²⁺ nor Zn²⁺ ions could be included at the higher pH values owing to oxidation or precipitation, respectively. Thus, in total, four selection experiments were performed.

In both selection experiments that used the DNA-C₃-NH₂ substrate, DNA-catalyzed activity was observed. After eight rounds (conditions A; final round only 2 hours of incubation time) or seven rounds (conditions B), 20% or 13% ligation activity was observed (see all selection progressions in Figure S2 in the Supporting Information). Individual deoxyribozymes were cloned (see Figure S3 for sequences) and observed to catalyze amine-DNA conjugation by reaction of the amino group with 5'-Imp.

Seven deoxyribozymes were found for conditions A, with *k*_{obs} values of 0.2–1.2 h⁻¹ and up to 85% yield (Figure 3 and Figure S4). Among these seven DNA enzymes, three different metal ion dependencies were observed. The 8DW115 deoxyribozyme and four other deoxyribozymes each require the presence of Mn²⁺, with little or no activity (less than 2%) in the presence of only Mg²⁺ and Zn²⁺ ions. The 8DW120 deoxyribozyme has optimal catalytic activity with Mn²⁺ but separately has some activity with the combination of Mg²⁺ and Zn²⁺. Finally, the 8DW113 deoxyribozyme requires both Mn²⁺ and Zn²⁺ ions, whereas Mg²⁺ is not necessary for catalysis. All seven deoxyribozymes were assayed at pH 7.2, 7.5, and 7.8. All deoxyribozymes except 8DW120 had optimal yield at pH 7.5 (but with still generally substantial yields at pH 7.2 and pH 7.8), whereas 8DW120 had slightly higher activity at pH 7.8 (Figure S5). Separately, seven Mg²⁺-dependent deoxyribozymes were identified for conditions B, with *k*_{obs} values of approximately 0.03 h⁻¹ and yields of 30–40% in 48 hours (Figure S6). The optimal Mg²⁺ ion concentration for each of these deoxyribozymes was approximately 30 mM (Figure S7). Each deoxyribozyme was assayed at pH values of 8.0–10.0 in 0.5-unit increments, and in each instance, optimal activity was observed at either pH 8.5 or pH 9.0 (Figure S6).

The two selection experiments that used the DNA-HEG-CKA substrate also led to substantial DNA-catalyzed activity. After nine rounds (conditions A) or 14 rounds (conditions B), 19% or 14% ligation activity was observed. A single deoxyribozyme, 9DT105, emerged from conditions A to catalyze the reaction of the Lys amino group of DNA-HEG-CKA with 5'-Imp, with *k*_{obs} 0.10 h⁻¹ and 50% yield (Figure 4). 9DT105 requires both Mn²⁺ and Zn²⁺ ions, whereas Mg²⁺ is dispensable. The yield of the reaction catalyzed by 9DT105 was substantially reduced at pH values below 7.5; for example, just 2% yield of product was obtained in 48 hours at pH 7.2 (Figure S8). A second deoxyribozyme from this selection, 9DT114, catalyzed a reaction with *k*_{obs} 0.17 h⁻¹ (Figure S9). However, the DNA anchor alone (lacking both HEG tether and CKA peptide) was sufficient for reactivity, surprisingly indicating that the nucleophile for

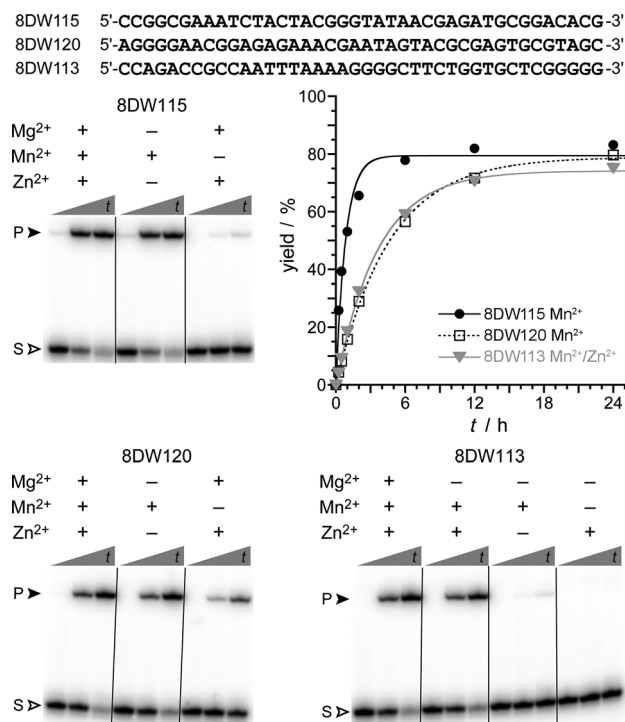


Figure 3. Deoxyribozymes for reaction of the DNA- C_3 - NH_2 substrate with 5'-ImpDNA. The initially random-sequence region (N_{40}) of each deoxyribozyme is shown (top). Representative PAGE images are shown for single-turnover assays of deoxyribozymes identified from conditions A (pH 7.5, Mg/Mn/Zn, deoxyribozymes 8DW115, 8DW120, and 8DW113).^[10] S = DNA- C_3 - NH_2 substrate; P = ligation product; t = 30 s, 2 h, and 24 h. The plot shows the change in ligation product yield with time for the 8DW115, 8DW120, and 8DW113 deoxyribozymes in the presence of the indicated metal ions. Incubation conditions: 50 mM ($-Zn^{2+}$) or 70 mM ($+Zn^{2+}$) HEPES, pH 7.5, with Na^+ (150 mM) and the indicated combinations of Mg^{2+} (40 mM), Mn^{2+} (20 mM), and Zn^{2+} (1 mM) at 37°C. See Figure S4 for comprehensive metal ion dependence and k_{obs} values and Figure S5 for kinetic plots at various pH values for all seven 8DW1 deoxyribozymes.

the 9DT114-catalyzed reaction with 5'-Imp is on the DNA anchor itself. More detailed investigation revealed that the nucleophile is the C^4 - NH_2 group of a particular deoxycytidine (Figure S9). Separately, six deoxyribozymes were found for reactivity of DNA-HEG-CKA under conditions B, with k_{obs} values of approximately $0.05\ h^{-1}$, yields up to 50% in 48 hours (Figure S10), and optimal Mg^{2+} ion concentration of more than 50 mM (Figure S11). For all six deoxyribozymes, the yield increased between pH 8.0–10.0 (Figure S10).

MALDI mass spectrometry corroborated product structures for several representative deoxyribozymes (Figure S12). The identity of each newly formed phosphoramidate (P–N) linkage was consistent with the observed acid sensitivity (Figure S13).^[2b,d,11] Negative control experiments were consistent with nucleophilic reactivity of the amine and electrophilic reactivity of 5'-Imp (Figure S14).

The 21 deoxyribozymes collectively obtained from the four different selection experiments (excluding 9DT114) were each separately assayed with four substrates, two of which were the selection substrates depicted in Figure 2a (for

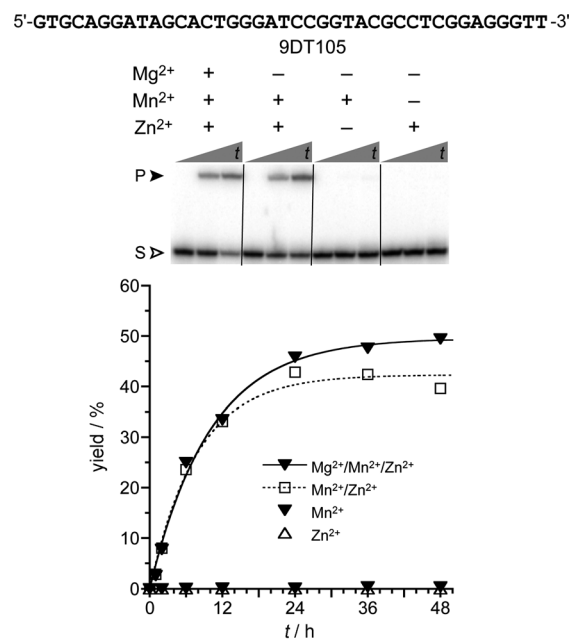


Figure 4. 9DT105 deoxyribozyme for reaction of the Lys of the DNA-HEG-CKA substrate with 5'-ImpDNA, identified from conditions A (pH 7.5, Mg/Mn/Zn). The initially random-sequence region (N_{40}) of 9DT105 is shown (top). A representative PAGE image is shown for single-turnover assays of the 9DT105 deoxyribozyme (center). S = DNA-HEG-CKA substrate. P = ligation product. The plot shows the change in ligation product yield with time for the 9DT105 deoxyribozyme in the presence of the indicated metal ions (bottom). t = 30 s, 6 h, and 48 h. Incubation conditions: 50 mM ($-Zn^{2+}$) or 70 mM ($+Zn^{2+}$) HEPES, pH 7.5, with Na^+ (150 mM) and the indicated combinations of Mg^{2+} (40 mM), Mn^{2+} (20 mM), and Zn^{2+} (1 mM) at 37°C. See Figure S8 for pH dependence.

simplicity now omitting the prefix “DNA-” for the DNA anchor): C_3 - NH_2 , HEG- NH_2 , C_3 -CKA, and HEG-CKA. (The C_3 -CKA and HEG- NH_2 substrates have structures analogous to those in Figure 2a. For C_3 -CKA, the C_3 tether terminates in a thiol rather than an amine and is joined using a disulfide to CKA. For HEG- NH_2 , the HEG tether terminates in an amine rather than a thiol.) The purpose of these assays was to evaluate comprehensively the tether and peptide dependence of the various deoxyribozymes. The results reveal two distinct types of substrate preference, both of which are sensible based on the selection origins of the various deoxyribozymes (Figure 5).^[9b] The deoxyribozymes identified from selection using the C_3 - NH_2 substrate under either incubation conditions A (deoxyribozymes designated 8DW1) or conditions B (7DX1) all have activity in the order C_3 - NH_2 > HEG- NH_2 > C_3 -CKA and HEG-CKA. Conversely, the deoxyribozymes selected using the HEG-CKA substrate under conditions A (9DT105) or conditions B (14DV1) all have higher activity with the Lys-containing substrates, HEG-CKA > HEG- NH_2 and C_3 -CKA > C_3 - NH_2 . The 9DT105 deoxyribozyme prefers the shorter-tethered peptide (C_3 -CKA > HEG-CKA), whereas each of the 14DV1 deoxyribozymes favors the longer-tethered peptide (HEG-CKA > C_3 -CKA). From these data, a key finding is that performing selection using

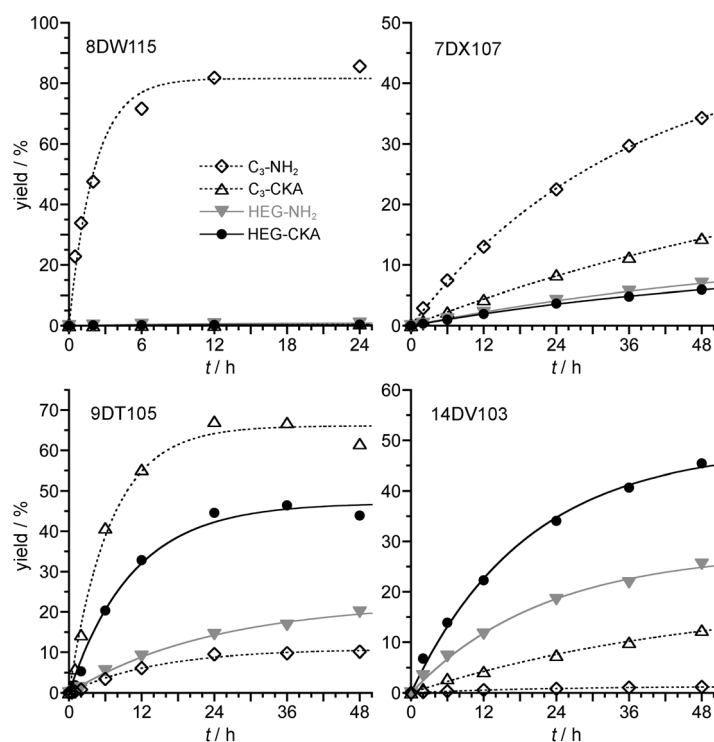


Figure 5. Plots of ligation product yield versus time for the 8DW115, 7DX107, 9DT105, and 14DV103 deoxyribozymes, showing the dependence of catalytic activity on substrate structure. The assays used substrates that have different tether lengths and amine environments. For the 8DW1, 7DX1, and 14DV1 deoxyribozymes, data for one representative catalyst is shown (see Figure S15, Figure S16, and Figure S17 for comprehensive data). Incubation conditions as given in Figure 3 and Figure 4.

the HEG-tethered substrate is necessary to achieve substantial DNA-catalyzed reactivity with that substrate.

9DT105 and the six 14DV1 deoxyribozymes were each assayed with the free (non-DNA-anchored) CKA tripeptide at up to 1 mM concentration. The unattached DNA anchor oligonucleotide was included to occupy the corresponding deoxyribozyme binding arm. In all cases, no Lys reactivity was observed (less than 1%; data not shown). This observation is unsurprising because the peptide was tethered to the DNA anchor oligonucleotide throughout the selection process (Figure 2). Therefore, the DNA sequences were never challenged to function in the absence of the tether. In other experiments, we have identified deoxyribozymes that do have some activity with free peptides,^[2e,8] although such activity was not always found.^[12] Overall, the rules are unclear for emergence of free peptide reactivity, suggesting the need for a strategy aimed specifically at this outcome. In a parallel study, we have established a new selection approach that enables the use of free, unanchored peptides directly during selection and thereby provides deoxyribozymes with useful yields and apparent K_m values, where K_m is the Michaelis constant for the DNA-peptide binding interaction.^[13] We anticipate that this new approach will be successful with Lys side chain reactivity of free peptide substrates in future experiments.

Rate enhancements for the various deoxyribozymes were estimated by comparing their k_{obs} values (Figure 3 and Figure 4) to the observed rate constants for appropriate background reactions (k_{bgd} ; Figure S18). Both conditions A (pH 7.5) and conditions B (pH 9.0) were evaluated. Using the random N_{40} pool in place of a catalytically active deoxyribozyme in the background assay, k_{bgd} values were calculated to be approximately 10^{-4} h^{-1} (under conditions A) and approximately two- to threefold higher (under conditions B). For conditions A, the DNA-catalyzed rate enhancement was up to 10^4 with the DNA- $\text{C}_3\text{-NH}_2$ substrate and up to 10^3 with the DNA-HEG-CKA substrate. For conditions B, the rate enhancements were as high as 10^2 for both substrates, with these more modest values largely reflecting that the deoxyribozymes from conditions B have lower k_{obs} values than do their counterparts from conditions A.

The selections performed at pH 7.5 each used a mixture of Mg^{2+} , Mn^{2+} , and Zn^{2+} ions, and the resulting DNA catalysts each require either Mn^{2+} or a combination of Mn^{2+} and Zn^{2+} for optimal activity. We reported a DNA-hydrolyzing deoxyribozyme that similarly requires a combination of Mn^{2+} and Zn^{2+} ions, although mutations could remove the Mn^{2+} dependence.^[14] Interestingly, none of the new deoxyribozymes found herein at pH 7.5 requires Mg^{2+} , although each deoxyribozyme identified by selection at pH 9.0 in the presence of Mg^{2+} ions alone (because Mn^{2+} and Zn^{2+} cannot be used at high pH values) requires Mg^{2+} . Understanding these various metal ion requirements, and indeed understanding all mechanistic aspects of the new deoxyribozymes, will require more detailed biochemical experiments, likely in the context of high-resolution structural information that is currently unavailable for any DNA catalyst.^[15]

Lys reactivity has never been observed previously with either DNA or RNA enzymes, including in our previous studies that successfully led to Tyr- and Ser-modifying deoxyribozymes.^[2a,b] This relative unreactivity of Lys using nucleic acid catalysts has been a surprising challenge. The unprotonated aliphatic amino group of Lys is comparable in nucleophilicity to the deprotonated phenolic OH of Tyr, and an amine is many orders of magnitude more nucleophilic than the nondeprotonated aliphatic OH of Ser;^[16] both of these considerations suggest that Lys should be rather reactive. Several observations are consistent with the collective picture that nucleic-acid-catalyzed nucleophilic reactions of nitrogen centers are difficult but achievable. DNA-catalyzed reductive amination involving a guanosine nucleobase N^2 -amine has been described,^[17] and an RNA-catalyzed reaction of a peptide N -terminal α -amino group was reported, even in the presence of a Lys side chain as a competing nucleophile.^[18] Herein, the emergence of both 9DT105 and 9DT114 deoxyribozymes from the same selection experiment indicates that DNA-catalyzed amine reactivity for the less preorganized DNA-anchored HEG-CKA substrate is sufficiently difficult to achieve, such that reactivity of an alternative nucleophile on the DNA anchor itself (for example a $\text{C}^4\text{-NH}_2$ group)^[19]

can instead be observed. In this context, we reemphasize our prior finding that with 5'-pppRNA as the electrophile, a phosphoramidate functional group reacted instead of a Lys amino group,^[2d] highlighting the relative unreactivity of an aliphatic amine when using a nucleic acid catalyst. We also note that uncatalyzed, DNA-templated polymerization of 5'-ImpDNA monomers by reaction with 3'-NH₂ groups is quite rapid (complete reaction in less than 1 hour; pH 7.5, 4 °C).^[20]

In summary, the key to successful DNA-catalyzed Lys reactivity was providing the more reactive 5'-phosphorimidazole (5'-Imp) electrophile, which was attacked by the Lys nucleophile despite the less preorganized selection architecture. These results reveal that the degree of deoxyribozyme-substrate preorganization is a less important design consideration for DNA catalysts than is the inherent reactivity of the electrophilic reaction partner, at least for nucleophilic amine reactions. The findings in this study provide important fundamental information to enable ongoing identification of DNA catalysts for covalent modification of peptide and protein substrates. Our efforts are particularly focused on biologically relevant modifications, such as acylation at Lys residues.^[4]

Experimental Section

DNA oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) or prepared by solid-phase synthesis on an ABI 394 instrument using reagents from Glen Research. All oligonucleotides and conjugates were purified by 7 M urea denaturing 20 % or 8 % PAGE with running buffer 1 × TBE (Tris (89 mM), boric acid (89 mM), and EDTA (2 mM), pH 8.3), extracted from the polyacrylamide with TEN buffer (Tris (10 mM), pH 8.0, EDTA (1 mM), NaCl (300 mM)), and precipitated with ethanol. Peptides were prepared by solid-phase synthesis using Fmoc-Rink-amide-MBHA resin as described.^[2e] Each peptide was coupled to the DNA anchor oligonucleotide by a disulfide bond with the N-terminal cysteine side chain as described.^[2e]

Full procedures for selection, cloning, and initial analysis of individual clones are provided in the Supporting Information.

The general single-turnover assay procedure for each deoxyribozyme was as follows. The DNA-anchored amine substrate was 5'-³²P-radiolabeled using γ-³²P-ATP and T4 polynucleotide kinase (Fermentas), using 10 × kinase buffer that lacks DTT (Tris (500 mM), pH 7.6, MgCl₂ (100 mM), and spermidine (1 mM)) for disulfide-linked oligonucleotide-peptide conjugates. A 10 μL sample containing 5'-³²P-radiolabeled substrate (0.2 pmol), deoxyribozyme (10 pmol), and 5'-Imp substrate (30 pmol) was annealed in HEPES (5 mM), pH 7.5, NaCl (15 mM), and EDTA (0.1 mM) for conditions A; or CHES (5 mM), pH 9.0, NaCl (15 mM), and EDTA (0.1 mM) for conditions B, by heating at 95 °C for 3 min and cooling on ice for 5 min. The DNA-catalyzed reaction was initiated by bringing the sample to 20 μL total volume, containing HEPES (70 mM), pH 7.5, ZnCl₂ (1 mM), MnCl₂ (20 mM), MgCl₂ (40 mM), and NaCl (150 mM) for conditions A; or CHES (50 mM), pH 9.0, MgCl₂ (40 mM), and NaCl (150 mM) for conditions B. The sample was incubated at 37 °C. At appropriate time points, 2 μL aliquots of reaction mixture were quenched with 5 μL of stop solution (formamide (80 %), 1 × TBE [Tris (89 mM), boric acid (89 mM), and EDTA (2 mM), pH 8.3], EDTA (50 mM), bromophenol blue (0.025 %), xylene cyanol (0.025 %)). Samples were separated by 20 % PAGE and quantified using a Phosphorimager. k_{obs} values were obtained by fitting the yield versus time data directly to first-order kinetics, $\text{yield} = Y(1 - e^{-k_{\text{obs}}t})$, where $k = k_{\text{obs}}$ and Y = final yield. Each

k_{obs} value is reported with error calculated as the standard deviation from the indicated number of independent determinations. When the k_{obs} value was sufficiently low such that an exponential fit was not meaningful, the initial points were fit to a straight line, and the k_{obs} value was taken as the slope of the line.

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